Bacillus cereus: Aerobic Growth Kinetics

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ABSTRACT

Three strains of *Bacillus cereus* were cultured in brain heart infusion medium aerobically under conditions of variable temperature (5 to 42°C), sodium chloride concentrations (0.5-5%), pH (4.5 - 7.5), and sodium nitrite concentration (0 mg/L - 200 mg/L) to simulate conditions of normal and adverse food storage. Cultures were sampled at selected times, and plate counts were used to calculate growth curves under each condition. None of the three strains grew at 5°C, but growth did occur slowly at 8°C, and was most rapid at a temperature of 37°C. Growth occurred in media without additives in all pH's examined between 8 and 42°C. Decreasing the pH and increasing levels of sodium chloride and sodium nitrite increased the lag phase and generation times of the organism.

Bacillus cereus is a gram (+), catalase (+), facultative, spore-forming rod implicated in foodborne disease outbreaks following consumption of various food products. Both diarrheal and emetic toxins may be produced by the growth of the organism in foods, the former generally in protein foods such as meat and dairy products, and the latter in high carbohydrate foods such as boiled rice (*13*). Growth of *B. cereus* in foods may occur from transfer of vegetative cells from soil and other contaminated surfaces or from outgrowth of spores that survived minimal processing conditions. This study examines the aerobic growth patterns of *B. cereus* vegetative cells under conditions of defined temperature, pH, level of sodium chloride, and level of sodium nitrite to develop a database on growth of vegetative cells in conjunction with a corresponding database on germination and outgrowth of the spores for use in design of safe food processing and storage recommendations.

MATERIALS AND METHODS

Bacterial strains

Three strains of *B. cereus* (F4810, an emetic toxin-producing strain isolated by R. Gilbert from cooked rice; B4AC, a diarrheal toxin-producing stain isolated by D. Mossel from pea soup; and T, reference strain (all furnished by F. Busta) were maintained on tryptic soy agar slants at 5°C until used. For the mixed strain culture, each strain was grown individually in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI) at 28°C for 18 h achieving ca. 10^8 CFU/ml. The three strain aliquots were combined and diluted to furnish an actively growing mixed vegetative culture of ca 10^4 to 10^5 CFU/ml as the inoculant and to provide a broader database of growth date. Separate starter flasks were prepared for each experiment.

Growth conditions

BHI was used for all studies as the basal medium. Alterations in the media were produced by addition of the desired final content of acid or sodium chloride to half-final volumes of a 2X BHI preparation with subsequent dilution to final volume. Sample flasks (250-ml Erlenmeyer) containing 50 ml medium were prepared and autoclaved. Sodium nitrite solutions were prepared at a concentration of 25 mg/ml and were filter sterilized. The calculated amount of sterile nitrite was added where needed to the BHI broth following autoclaving and cooling. Since BHI contains 0.5% sodium chloride, this salt concentration was the base level for these experiments. The pH of media was adjusted with addition of concentrated HCl or KOH prior to autoclaving and was checked following sterilization.

Growth temperature

Sterile test media were inoculated with 100 µl of the 10^5 CFU/ml mixed *B. cereus* culture to furnish a concentration of 10^3 CFU/ml at zero time. Triplicate flasks were used for each cultural condition and were warmed or cooled before inoculation to temperatures near their subsequent incubation temperatures. Flasks were incubated aerobically with shaking (150 rpm) at 5, 8, 12, 19, 28, 37, and 42°C.

Experimental design

A factorial plus supplemental composite experimental design was used for these studies. Additional experiments were added to the initial design at the extremes of temperature or pH, since previous experience indicated quantifying certain variable interactions required further data (*4*). Variables of incubation temperature, initial pH, sodium chloride level, and sodium nitrite level of the media were chosen to simulate conditions present in foods under adequate and abuse storage environments. Inoculated flasks were sampled at selected times appropriate for the various temperatures to determine level of growth at times corresponding to initial inoculum, lag phase, log-growth phase, and maximum population density phase. Sampling times were minimized to prevent temperature fluctuations.

Aliquots from the experimental flasks were diluted into 0.1% peptone (Difco) water and surface plated on tryptic soy agar (Difco)

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with a Spiral Plater Model D (Spiral System Instruments, Bethesda, MD). Plates were incubated for 18 h at 22°C rather than at higher incubation temperatures to minimize colony spreading. Colonies were counted with a Laser Bacteria Colony Counter (Model 500 A) with the Computer Assisted Spiral Bio-Assay data processor (Spiral Systems Instruments) and cross-checked frequently with manual counting. This procedure furnishes results with *B. cereus* equivalent to manual plate counts (*14*). Arithmetic counts were converted to $\log_{10}$ CFU/ml values.

A total of 226 growth curves were generated with individual curves fitted using the Gompertz equation (Table 1) (*4,7,22*) in conjunction with Abacus, a nonlinear regression program for bacterial growth curve fitting (W. Damert, Eastern Regional Research Center, personal communication).

RESULTS

Cultural conditions used in this study and the calculated values for lag-phase duration (LAG), generation or doubling time (GT), and maximum population density (MPD) (means of triplicate determinations) are shown in Table 2.

The three strains of *B. cereus* used in this study did not grow at 5°C and had a minimum growth temperature of 8°C. Incubation temperatures of 6 and 7°C (data not shown) did not support growth. A "no growth" determination was indicated by a continued drop in the microbial concentration with time to an eventual nondetectable level (<21 CFU/ml). Sampling was continued for 3 d following nondetectable counts to ensure that the organisms were nongrowing, and plates were stored at room temperature and recounted after 24 h to determine any spore outgrowth.

With the exception of the growth at 8°C, most samples showing growth reached MPD between log 7 to log 9 CFU/ml. Only minor effects were noted in this experimental parameter as a result of media pH, sodium chloride, or nitrite modifications. As expected, conditions favoring growth (no additives, pH $\geq$ 6.0, temperatures > 19°C) reduced the lag-phase times and the GT. The most rapid growth rate in this study occurred at 37°C, pH 6.75, with no additives, with the minimum lag time of 0.91 h and a doubling time of 0.23 h. Under these conditions, an initial level of 10^3 CFU/ml attained levels of 10^6 and 10^9 CFU/ml at 3.5 and 6.1 h, respectively. Growth was almost as rapid in media at pH 6.0 with no additives. In contrast, the slowest growth to that MPD level occurred at 8°C in samples (pH 6.50, 0.5% sodium chloride, and 0 mg/L nitrite) where the calculated times to reach 10^6 and 10^9 levels were 13 and 13.7 d, respectively, with the lag-phase time of 301.8 h. These data indicate the importance of good refrigeration for foods containing *B. cereus* vegetative cells.

Plots of calculated lag-phase values versus GT values for the combinations of salt and nitrite additives (not shown) also indicate that lowered growth temperatures or high salt levels lead to the greatest increase in those two parameters. Nitrite as the only additive has lesser effect.

DISCUSSION

Bacillus cereus has been recognized as a potential foodborne intoxicant, but most reports have focused on the emetic toxin usually quite evident after consumption of contaminated cooked rice (*8,12,13*). The diarrheal toxin, because it

TABLE 1. *Gompertz equation.*

The Gompertz equation is $L(t) = A + C \exp\{-\exp(-B[t-M])\}$,

where L(t) is the $\log_{10}$ of the CFU/m at time t;
A= $\log_{10}$ of the initial level of bacteria at time 0;
C = number of log cycles of growth;
B = relative growth rate at M (time of maximal growth rate).

The four terms - A, B, C, and M - may be transformed into:

Exponential growth rate (EGR), expressed as $\log_{10}$ (CFU/ml)/h, and equivalent to B*C/e,
where e is the natural logarithm;

Generation time (GT) or doubling time, expressed in h, and equivalent to $\log_{10}$ of 2*e/B*C;

Lag-phase duration (LAG), expressed in h, and equivalent to M - 1/B; and

Maximum population density (MPD), expressed as $\log_{10}$ CFU/ml at the end of growth phase,
and equivalent to A + C.

TABLE 2. *Growth conditions and calculated growth kinetic parameters.*

Temp. (°C)	pH	NaCl (%)	$NaNO_2$ (ppm)	Lag time (h)	GT (h)	MPD ($\log_{10}$)
5	6.00	2.5	100	No growth		
	6.25	0.5	0	No growth		
	6.50	0.5	0	No growth		
	7.00	0.5	0	No growth		
	7.00	2.5	0	No growth		
	7.50	0.5	0	No growth		
8	5.50	0.5	0	No growth		
	5.75	0.5	0	No growth		
	6.00	0.5	0	No growth		
	6.50	0.5	0	301.81	1.34	8.20
	6.50	0.5	100	No growth		
	6.50	0.50	200	No growth		
	6.50	1.50	0	No growth		
	6.50	1.50	200	No growth		
	7.00	0.50	0	94.64	2.45	6.00
12	5.25	0.5	0	No growth		
	5.25	0.5	200	No growth		
	5.25	1.5	50	No growth		
	5.25	1.5	150	No growth		
	5.25	2.0	0	No growth		
	6.00	0.5	0	23.05	6.31	7.71
	6.25	0.5	0	27.92	5.17	7.47
	6.50	0.5	0	22. 19	7.04	7.36
	6.75	1.5	50	81.28	5.99	8.84
	6.75	1.5	150	56.76	5.91	7.19
	6.75	2.0	0	22.98	5.36	7.20
	6.75	3.5	50	136.09	6.05	8.39
19	4.50	2.5	100	No growth		
	5.25	0.5	0	4.70	1.55	9.00
	5.50	0.5	50	2.93	1.08	8.95
	5.75	2.5	50	18.06	2.56	6.86
	6.00	0.5	0	6.50	0.32	9.42
	6.00	0.5	50	7.35	0.34	8.84
	6.00	0.5	100	8.77	0.69	8.66
	6.00	0.5	200	10.29	1.50	8.57
	6.00	1.5	0	10.64	1.25	9.36
	6.00	2.5	100	16.86	1.83	8.29